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# Establishment of Genome-edited Human Pluripotent Stem Cell Lines: From Targeting to Isolation

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## Abstract

Genome-editing of human pluripotent stem cells (hPSCs) provides a genetically controlled and clinically relevant platform from which to understand human development and investigate the pathophysiology of disease. By employing site-specific nucleases (SSNs) for genome editing, the rapid derivation of new hPSC lines harboring specific genetic alterations in an otherwise isogenic setting becomes possible. Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 are the most commonly used SSNs. All of these nucleases function by introducing a double stranded DNA break at a specified site, thereby promoting precise gene editing at a genomic locus. SSN-mediated genome editing exploits two of the cell's endogenous DNA repair mechanisms, non-homologous end joining (NHEJ) and homology directed repair (HDR), to either introduce insertion/deletion mutations or alter the genome using a homologous repair template at the site of the double stranded break. Electroporation of hPSCs is an efficient means of transfecting SSNs and repair templates that incorporate transgenes such as fluorescent reporters and antibiotic resistance cassettes. After electroporation, it is possible to isolate only those hPSCs that incorporated the repair construct by selecting for antibiotic resistance. Mechanically separating hPSC colonies and confirming proper integration at the target site through genotyping allows for the isolation of correctly targeted and genetically homogeneous cell lines. The validity of this protocol is demonstrated here by using all three SSN platforms to incorporate EGFP and a puromycin resistance construct into the AAVS1 safe harbor locus in human pluripotent stem cells.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/53583/>

## Introduction

Genome editing technologies are rapidly evolving into standard tools for molecular and cell biology<sup>1</sup>. Genetic engineering of human pluripotent stem cells (hPSCs) is of particular interest as hPSCs represent a self-renewing source of genetically intact primary human cells. hPSCs can be differentiated into various cell types for disease modeling or as a source for transplantation therapies<sup>2,3</sup>. Demonstrated here is a protocol that utilizes three different types of site-specific nucleases (SSNs) in conjunction with endogenous DNA repair mechanisms for targeted integration of a reporter construct at the AAVS1 locus. After transfection of SSNs into hPSCs, we demonstrate how to isolate isogenic cell populations harboring the reporter.

The ability to manipulate genomes, specifically pluripotent stem cell genomes, using SSNs is not a new phenomenon as the utility of zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) for gene editing was demonstrated several years ago<sup>4-10</sup>. However, with the advent of *S. pyogenes* CRISPR/Cas9 technology<sup>11-13</sup>, gene editing has become widely accessible<sup>14</sup>. All SSNs introduce a double stranded DNA break (DSB) at the specified target site<sup>1,4,5,11</sup> that is repaired by endogenous cellular mechanisms using either non-homologous end-joining (NHEJ) or homology directed repair (HDR)<sup>15</sup>. NHEJ is error-prone and can introduce frame shift mutations resulting in loss of gene function, while HDR allows for novel elements to be introduced through the co-transfection of a repair template with the SSN. While the underlying principles of DNA repair that facilitate gene editing are thought to be largely the same for each SSN, some differences between the platforms can be noted. *De novo* design of ZFNs allows flexibility and nuclease optimization<sup>16</sup>, however the use of publicly available assembly libraries and screening tools to design individual ZFNs can be time consuming. Once the desired locus for ZFN-mediated targeting is determined, ZFN pairs can be designed with the online tool ZiFit<sup>17</sup>. After design, ZFNs can be modularly assembled through several rounds of plasmid cloning<sup>18</sup>. Alternatively, there are many commercially available, pre-validated ZFNs<sup>19</sup>. TALE nucleases can also be designed using online tools and publically available components<sup>17,20</sup>. For example, TALENs can be rapidly assembled from blocks of five TALE repeats, through FLASH assembly<sup>21</sup> or using PCR based hierarchical Golden Gate assembly<sup>22</sup>. Ease of SSN design and speed of construction using CRISPR/Cas9 have made genome editing a widely accessible tool. The short guide RNA-mediated targeting of CRISPR/Cas9 also allows for multiplexing of guide RNAs to target several loci with a single construct<sup>14</sup>. The design of Cas9 for gene editing requires only the identification of a protospacer adjacent motif (PAM; an NGG trinucleotide for *S. pyogenes* Cas9) proximal to the target locus. By inserting an oligonucleotide corresponding to the 20 base pairs 5' of the PAM into the px330 plasmid<sup>14</sup>, the construct can be assembled in one cloning step. In addition to

*S. pyogenes* Cas9, Cas9 from *N. meningitidis* (NmCas9) that recognizes a 5'-NNNNGATT-3' (PAM) has been shown to allow for efficient gene-editing in hPSCs<sup>23</sup>.

In addition to the differences in ease of SSN design, each platform has specific properties. For example, ZFNs and TALENs utilize the FokI nuclease domain, which generates a four nucleotide 5' overhang<sup>24</sup> while Cas9 is thought to generate mostly blunt ended DSBs. ZFNs, TALENs, and Cas9 differ in their protein stabilities, on-off rate on target DNA, and mode of DNA scanning, all of which could theoretically result in small differences in the editing outcome<sup>1</sup>. While further studies will be required to fully understand the consequences of these differences, we describe here a protocol that is highly robust across all three platforms and can be used to readily generate genetically modified hPSCs.

Regardless of SSN choice, electroporation is a robust procedure to transfect SSNs and homology repair templates into hPSCs<sup>25</sup>. The number of surviving colonies after selection for antibiotic resistance depends on locus-specific parameters and the editing strategy (e.g., size of transgenic insert and mode of selection). The protocol described here usually results in about 150-400 single-cell derived colonies.

Gene-editing at the AAVS1 locus using this protocol has previously been used to demonstrate the effectiveness of SSNs<sup>4,5</sup>. The AAV-CAGGS-EGFP repair template uses a gene trap strategy to confer puromycin resistance in a locus specific manner. Briefly, the repair template contains a splice acceptor site upstream of the promoterless puromycin resistance cassette. Upon correct integration into the first intron of the PPP1R12C gene at the AAVS1 locus, the resistance cassette is expressed from the edited gene's promoter. The robustness of this specific AAVS1 assay allows us to compare the efficiency of each SSN platform.

Gene editing using SSNs is powerful given the ability to disrupt and/or alter theoretically any gene. Applying this strategy to hPSCs provides versatility as hPSCs can be subsequently differentiated into a multitude of human cell types such as neurons<sup>26</sup>, hepatocytes<sup>27</sup>, and cardiomyocytes<sup>28</sup>. Additionally, the use of patient-derived induced pluripotent stem cells allows the repair or introduction of known disease-causing mutations in a patient-specific genetic background<sup>29</sup>, providing a platform from which to investigate disease mechanisms and test therapeutics using a patient's own cells<sup>30</sup>. In summary, gene editing in hPSCs is an efficient and versatile approach for investigating the basic biology of human development and disease<sup>31</sup>.

## Protocol

The procedures described in this manuscript were reviewed and approved by the UC Berkeley Stem Cell Research Oversight Committee.

### 1. Prepare Stem Cells for Editing

- Grow and culture human pluripotent stem cells (hPSCs) on a 6-well plate containing  $2.4 \times 10^6$  cells/plate of mitomycin C-inactivated mouse embryonic fibroblast (MEF) feeders grown on gelatin<sup>32</sup>. Maintain hPSCs in 3 ml of human embryonic stem cell media per well (hESC media) and grow in a 37 °C incubator with 3% O<sub>2</sub>/5% CO<sub>2</sub>.  
Note: For the success of this protocol, it is not necessary to maintain hPSCs in a low-oxygen incubator; however it is important to note that hPSCs proliferate faster in a high O<sub>2</sub> environment, so times and cell numbers should be adjusted accordingly. It should also be noted that hPSCs maintained in low oxygen have lower rates of spontaneous differentiation<sup>33</sup>.
  - To make 500 ml hESC media combine 380 ml DMEM/F12, 75 ml Fetal Bovine Serum (FBS), 25 ml KnockOut Serum Replacement (KSR). Add Glutamine (1 mM final concentration), 5 ml 100x Non-essential Amino Acids, 100 units/ml Penicillin-Streptomycin (P/S), basic Fibroblast Growth Factor (bFGF) (4 ng/ml final concentration), and 2-mercaptoethanol (5.5 μM final concentration).
- Change media by removing entire volume of media (3 ml) using a glass pipette and vacuum. Replace with 3 ml warm hESC media using a serological pipette. Repeat media change every day until hPSCs are approximately 50% confluent (Day -1).
- One day before targeting (Day -1), change hESC media, removing the old media and adding warm hESC media supplemented with 10 μM Y-27632.
- Also on Day -1, prepare one to two 6-well or 10 cm plates of drug resistant MEF feeder cells from DR4 mice ( $2.4 \times 10^6$  cells/plate)<sup>34</sup>.  
Note: In general, 6 well plates are advantageous over 10 cm plates, as they accommodate more media. 6 well plates also ensure that different clones from different wells are independent. However, a 10 cm plate will allow easier picking, depending on the microscope available for this process.

### 2. Editing Pluripotent Stem Cells

- Prepare transfection solutions by pipetting 5 μg each of ZFN 1 and 2, TALEN 1 and 2, or 15 μg of the CRISPR/Cas9 px330 encoding plasmid (**Figure 1**) into a 1.5 ml tube. Pipette 30 μg of the repair plasmid into this tube as well. Finally, pipette 1x Phosphate Buffered Saline (PBS) into the tube to bring the volume up to 300 μl.  
Note: Prepare plasmids as a midiprep (any kit is suitable) with a minimum concentration of 300 ng/μl in order to keep the total volume of the transfection solution under 300 μl. It is not necessary to perform phenol/chloroform extraction, to linearize the plasmid, or to use an endotoxin free plasmid prep kit.
- Remove media from hPSCs using a glass pipette and vacuum. Pipette 2 ml warm 1x PBS into each well to wash the cells.
- Remove the PBS immediately using a glass pipette and vacuum. Add 0.5 ml 0.25% Trypsin-EDTA solution directly onto cells in each well. Place in the incubator (37 °C/5% CO<sub>2</sub>/3% O<sub>2</sub>) for approximately 10 min. or until feeder layer starts to lift off the plate.
- Add 2 ml warm esWash media (470 ml DMEM/F12, 25 ml FBS, 100 units/ml P/S) to each well to stop the trypsin reaction.
- Ensure that feeder cells come off as a sheet. Pipette the contents of each well into a single 50 ml conical tube, combining all wells. Triturate cells using a 10 ml serological pipette. It is not necessary to break up the feeder layer; hPSCs will come off of the feeder layer by gentle trituration. There should be about 15 ml of the cell suspension.
- Add 25 ml of esWash media to the tube to bring the volume up to 40 ml total. Allow large feeder chunks to settle at the bottom of tube for 1-2 min. Remove the supernatant (~38 ml) from the tube using a serological pipette and deposit into a new 50 ml conical tube.

7. Spin down for 5 min at 190 x g. Remove the supernatant from the tube using a glass pipette and vacuum. Be sure to not disturb the cell pellet. Resuspend the cells in 500  $\mu$ l 1x PBS. Combine with plasmid transfection solution prepared earlier. Count the cells at this step. Use 5-10 million cells per electroporation.
8. Pipette the entire 800  $\mu$ l suspension into a 4 mm electroporation cuvette, place on ice for 3-5 min. Electroporate cells using the exponential program on the electroporation system with the following parameters: 250 V, 500  $\mu$ F,  $\infty$  resistance, and 4 mm cuvette size. After electroporation, place cuvette back on ice for 3 min.  
Note: Observe the time constant of the electroporation on the electroporation system. The time constant varies with cell number and DNA purity. Successful transfections usually have a time constant between 10-14 msec when using the listed conditions and a gene pulser II. Lower electroporation efficiencies can occur when time constants varies from these values.
9. Resuspend electroporated cells in 18 ml warm hESC Media supplemented with 10  $\mu$ M Y-27632. Plate 3 ml of this single cell suspension into each well of a 6 well plate with DR4 feeder cells. Return to incubator (37 °C/5% CO<sub>2</sub>/3% O<sub>2</sub>).

### 3. Selection of Positive Colonies

1. Day 2, remove all media using a glass pipette and vacuum. Replace with 3 ml of warm hESC media supplemented with 10  $\mu$ M Y-27632. Day 3, remove all media using a glass pipette and vacuum. Replace with 3 ml of warm hESC media without including Y-27632. Day 4, remove all media using a glass pipette and vacuum. Replace with 3 ml warm hESC media supplemented with antibiotics for selection. Return to incubator (37 °C/5% CO<sub>2</sub>/3% O<sub>2</sub>).  
Note: The type of antibiotic used will depend on the resistance cassette included in the repair template. When working with WIBR#3 hPSCs (NIH registry 0079), 0.5  $\mu$ g/ml puromycin, 70  $\mu$ g/ml G418 (geneticin), and 35  $\mu$ g/ml hygromycin have been used successfully. Concentrations for selection should be determined empirically by establishing the minimal concentration of antibiotic needed to kill wild-type cells within approximately one week.
2. Days 5-12, change media daily, replacing old media each time with warm hESC media supplemented with antibiotics.  
Note: Expect a large amount of cell death. Individual colonies will become apparent around day 8-10. Regular hESC media without antibiotics can be used after 12-14 days of continuous selection. If cell density is high or cell death is slow, acidification of the media should be avoided and it may be necessary to increase the media volume (up to 4-5 ml) during the first few days of selection.

### 4. Picking Selected Colonies (Day 12-14)

1. Observe colonies on day 12-14. Observe the colonies that are ready to pick on a dissection microscope and ensure that they do not contain cells that are starting to differentiate. The approximate size should be 800-1,200  $\mu$ m. If colonies reach this size before day 12, it is recommended that they should be picked then.  
Note: For each targeting experiment, pick as many colonies as necessary to ensure the desired genotype is isolated. As an example, AAVS1 editing with the AAV-CAGGS-EGFP repair template has shown consistently robust integration, and requires only 12-24 colonies to be picked to obtain approximately 5-10 heterozygously targeted clones. Other targeting experiments may require more colonies to be picked (**Table 1**). The frequency of correct targeting events depends on factors such as the efficiency of the SSN to introduce a DSB, the size of insert, and the selection strategy. In the case of the gene trap approach for the AAVS1 locus presented here, the selection marker is only expressed when correctly integrated at the target site, reducing the number of colonies required to obtain a properly targeted clone.
2. One day before picking, prepare 12-well plates of MEFs ( $2.4 \times 10^6$  cells/plate, one well will be used for each colony picked).
3. On the day of picking, remove all media from the 12 well MEF plates using a glass pipette and vacuum and replace with 1 ml hESC media. Also on day of picking, change hESC media on the 6 well plates that are going to be picked.
4. Pull glass pipettes for mechanical dissociation of individual colonies from the feeder layer. Lower the pipettes over an in-hood Bunsen burner and softened at the expansion point until glass is malleable. Quickly remove from flame and pull the pipette apart creating an angular point. Break the point approximately 2 cm from the bent axis, leaving a narrow channel. Polish the end of the channel by exposing to flame for 1-2 sec.  
Note: Optionally pick colonies a p20 pipet tip, however, this may reduce the clonality of the culture, as the dull tip may dislodge greater amounts of cells from each colony into the media, potentially contaminating subsequent pickings.
5. Assemble picking device by taking a p1000 filter pipette tip and attaching a suction bulb to the narrow end. Insert the pulled glass pipette in the wide end.
6. Place a 6-well plate of hPSCs to be picked on the stage of a dissection microscope mounted in a tissue culture hood. Compress the bulb of the picking device and gently excise and cut an individual colony into 10-20 equally sized pieces, taking care not to release pieces into the media. Take excised hPSC pieces of the colony into the pipette by releasing the bulb. Try to take as little media as possible while transferring.
7. Transfer the individual colony to a single well of a 12-well MEF plate, compressing the bulb again directly into the well, releasing the now broken colony. Label each well to allow unique identification of single-cell derived clones. Repeat for as many colonies as necessary, changing the glass pipette each time.  
Note: Picking colonies may take some time to learn. It is recommended that the experimenter practice on some control cells before trying to isolate targeted colonies.
8. Return 12 well plates to the incubator (37 °C/3% O<sub>2</sub>/5% CO<sub>2</sub>), gently rocking the plate first to avoid accumulation of cells in the middle of each well.
9. The next day and each subsequent day, remove full volume of media using a glass pipette and vacuum and replace with 1.5 ml of warm hESC media until cells are about 50% confluent (this usually takes 10-12 days).
10. After 10-12 days, pick one to two colonies from each well and transfer to new 12-well MEF plates repeating steps 4.1-4.8 to generate a replica plate.
11. Extract DNA (see below) from the remaining colonies in each well of the original MEF plates.
  1. Remove media from all wells using a glass pipette and vacuum. Pipette 1 ml 1x PBS onto each well to wash cells. Remove PBS using glass pipette and vacuum. Pipette 250  $\mu$ l of cell lysis buffer (final concentrations in H<sub>2</sub>O: 10 mM Tris HCl, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 0.08 mg/ml Proteinase-K) onto each well. Place in incubator (37 °C/3% O<sub>2</sub>/5% CO<sub>2</sub>) O/N.

2. The next day, pipette contents of each well into individual 1.5 ml tubes. Pipette 250  $\mu$ l of isopropyl alcohol into each tube to precipitate the DNA. Shake tube vigorously. A white precipitate should be visible.
  3. Spin each tube down for 3 min at 13,000 rpm in a table top centrifuge. After spin down, discard supernatant by decanting into liquid waste. A small DNA pellet should remain stuck to the bottom of the tube. Pipette 250  $\mu$ l of 70% ethanol into each tube to wash the DNA pellet. Shake vigorously. Spin each tube down for 3 min at 13,000 rpm in a table top centrifuge.
  4. After spinning down, discard supernatant by decanting into liquid waste. A small DNA pellet should remain stuck to the bottom of the tube. Pipette out the remainder of the supernatant so there is no liquid left in the tube. Leave tube open to dry on benchtop for 5-10 min. After drying, resuspend DNA in 250  $\mu$ l TE buffer. Place at 37 °C for 6 hr to allow DNA to dissolve.
12. Genotype each sample using a pre-optimized PCR or southern blot strategy.  
 Note: For AAVS1 targeting using the AAV-CAGGS-EGFP repair template, the southern blot strategy can be found in Hockemeyer *et al.*, 2009<sup>4</sup>. A comprehensive southern blotting protocol can be found in Southern, 2006<sup>35</sup>. For multiplex PCR genotyping of the same experiment, primers and conditions can be found in **Table 2**<sup>36</sup>.
13. Discard wells that are not properly targeted and continue changing hESC media on properly targeted cells every day. Freeze down the cells when they are approximately 50% confluent, see below for freezing protocol.
1. One day before freezing, change hESC media, removing the old media and adding warm hESC media supplemented with 10  $\mu$ M Y-27632.
  2. On the day of freezing prepare 0.5 ml each of solution A and solution B per well of a 12-well plate that is being frozen down in two 15 ml conical tubes. Place solutions on ice. Solution A: 50% hESC media and 50% FBS; Solution B: 80% FBS and 20% Dimethyl Sulfoxide (DMSO).
  3. Dissociate hPSC colonies into single cells before freezing. Follow steps 2.2-2.8 to do this, scaling volumes accordingly. Fully resuspend cell pellet in 0.5 ml of solution A.
  4. Add 0.5 ml solution B to cell suspension, pipette up and down to make cell suspension homogenous. Take the total volume of the cell suspension (~1 ml) and deposit into a 2 ml cryotube. Screw cap on tightly.
  5. Place cryotube in a -80 °C freezer O/N. The day after freezing, remove frozen cells from -80 °C freezer and immediately place into liquid nitrogen tank for long-term storage.

## Representative Results

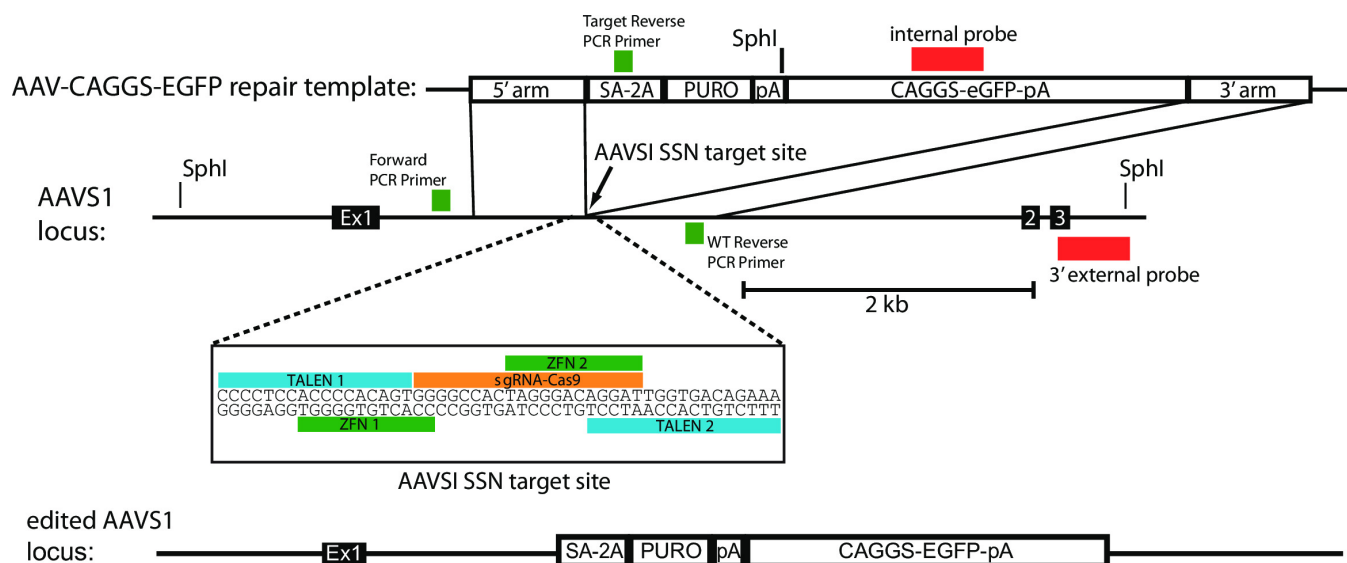
Here we demonstrate a protocol compatible with three different SSN platforms to create genetically engineered hPSC lines. We targeted WIBR#3 human embryonic stem cells at the AAVS1 locus using previously published ZFNs<sup>4</sup>, TALENs<sup>5</sup> and CRISPR/Cas9s<sup>37</sup> using a repair template that introduces an EGFP reporter and a puromycin resistance cassette<sup>4</sup>.

We cultured our hPSCs on MEFs for a cell culture workflow permitting the maintenance and expansion of undifferentiated hPSCs (**Figure 2**), that is also cost effective and scalable. If cells are grown longer than necessary there is a risk of increased differentiation, reducing the number of pluripotent cells that are transfected and consequently the number of correctly targeted hPSC colonies obtained.

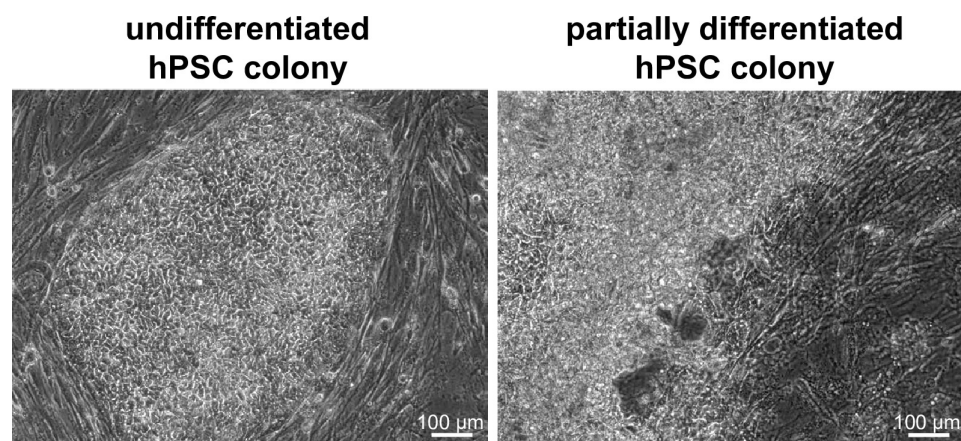
We electroporated  $5.0 \times 10^6$  cells per SSN platform and plated the cells from each targeting onto a single 6 well plate of DR4 MEFs. After selection, each platform resulted in EGFP-positive colonies (**Figure 3**) and a combination of untargeted, homozygously targeted and heterozygously targeted clones (**Figure 4A,B; Table 3**). Under the conditions presented here, we find that the AAVS1 TALENs resulted in the most EGFP-positive clones. The repair template used for this experiment consists of a splice acceptor site upstream of the EGFP reporter and puromycin resistance cassette. Using this "gene-trap" strategy (**Figure 1**), the construct should insert into the first intron of the AAVS1 locus, using the endogenous promoter to drive expression of the puromycin-resistance cassette. The lack of a promoter driving the expression of the puromycin resistance gene within the repair template should prevent expression in the event of random integration.

Therefore, we expect that all puromycin-resistant clones would be targeted at the AAVS1 site. It should be noted that a subset of clones carry aberrant integrations in the AAVS1 locus that are detectable by Southern blot using an internal probe, but not by most PCR strategies (**Figure 1; Figure 4C**)<sup>4</sup>. These integration events are most likely the result of heterologous targeting events leading to multiple integrations of the donor plasmid<sup>4</sup>. We picked 24 colonies from each SSN experiment and found that all platforms had very high targeting efficiencies and showed only minimal differences. As interrogated by PCR, CRISPR/Cas9 yielded the most correctly targeted clones while the TALEN platform had the most homozygously targeted clones (**Table 3**).

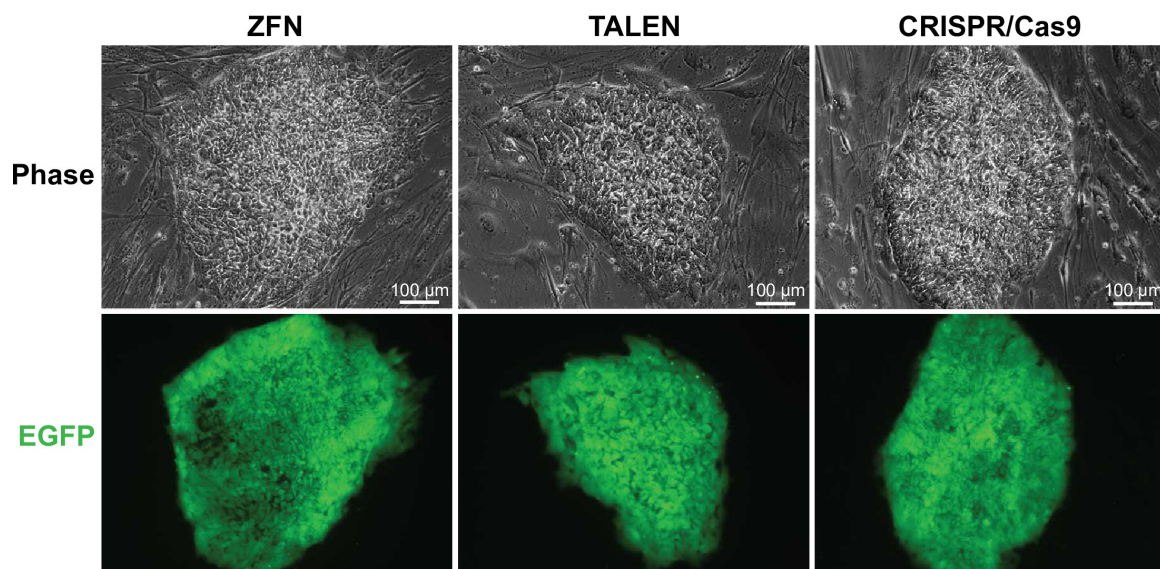




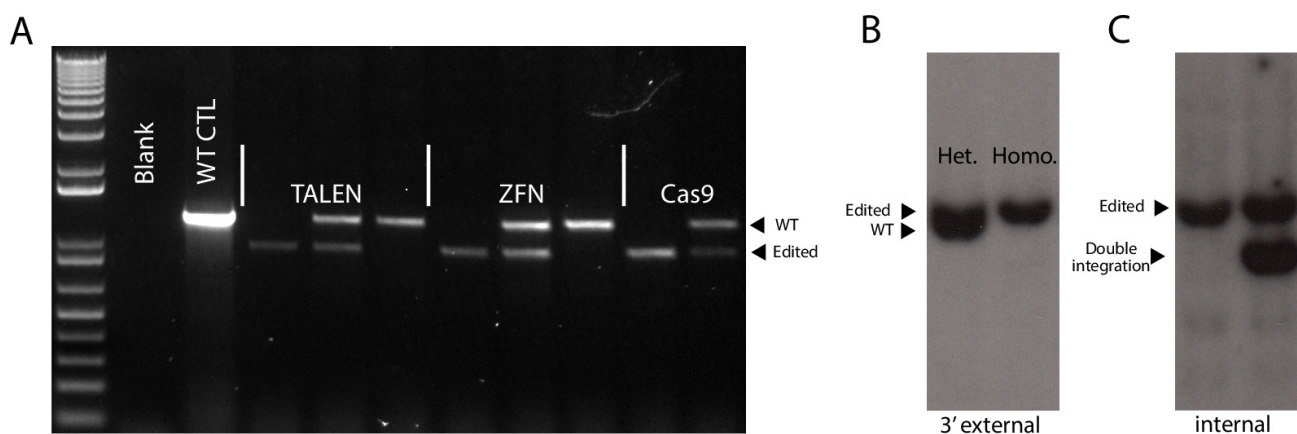
**Figure 1. Schematic of gene edited AAVS1 locus using the AAV-CAGGS-EGFP repair template.** Modified from Hockemeyer *et al.*, 2009. [Please click here to view a larger version of this figure.](#)



**Figure 2. Colonies of WIBR#3 cells.** Representative bright field images of colonies of WIBR#3 human embryonic stem cells prior to targeting. Note the lack of differentiation and the clear separation from the feeder layer in an ideal colony (left) as opposed to a non-ideal one (right). [Please click here to view a larger version of this figure.](#)



**Figure 3. EGFP-positive WIBR#3 cells.** Representative images of WIBR#3 cells targeted with an EGFP-expressing repair template at the AAVS1 locus. Images of representative colonies edited with ZFNs, TALENs and CRISPR/Cas9 are shown. [Please click here to view a larger version of this figure.](#)



**Figure 4. Genotyping strategies to confirm correct targeting.** (A) Representative PCR genotyping results showing untargeted, heterozygous and homozygous targeted clones across the three SSN platforms. WT CTL=wild-type control. (B) Representative Southern blot results showing a heterozygous targeted clone and a homozygous targeted clone detected with a 3' external probe. Fragment sizes: WT-6.5 kb, Edited-6.9 kb. (C) Representative Southern blot results showing a properly edited clone and a heterozygous clone with a non-random double-integration. Fragment sizes: Properly edited-6.9 kb, aberrant additional integration-5 kb. [Please click here to view a larger version of this figure.](#)

Study	Gene Targeted	Platform	Repair template type	# of clones picked	Targeting efficiency
Sexton <i>et al.</i> , 2014	TPP1	ZFN	GFP-Puro	unreported	unreported
Sexton <i>et al.</i> , 2014	TERT	ZFN	Hygromycin	unreported	unreported
Hockemeyer <i>et al.</i> , 2009	POU5F1	ZFN	GFP-Puro	31	39.0%
Hockemeyer <i>et al.</i> , 2009	PITX3	ZFN	GFP-Puro	74	14.9%
Hockemeyer <i>et al.</i> , 2011	POU5F1	TALEN	GFP-Puro	68	91.0%
Hockemeyer <i>et al.</i> , 2011	PITX3	TALEN	GFP-Puro	96	13.0%
Merkle <i>et al.</i> , 2015	VASA	Cas9	Reporter-Geneticin	139	94.0%
Merkle <i>et al.</i> , 2015	CRH	Cas9	Reporter-Geneticin	30	93.0%
Merkle <i>et al.</i> , 2015	HCRT	Cas9	Reporter-Geneticin	154	92.0%
Merkle <i>et al.</i> , 2015	HMX2	Cas9	Reporter-Geneticin	11	45.0%
Forster <i>et al.</i> , 2014	LRG5-Nterm	ZFN	GFP-Puro	unreported	30.0%
Forster <i>et al.</i> , 2014	LRG5-Cterm	ZFN	GFP-Puro	unreported	14.0%
Soldner <i>et al.</i> , 2011	SNCA	ZFN	Puro	96	1.0%

**Table 1. Description of other genes targeted using this method, with corresponding targeting efficiencies taken from previously published studies.**<sup>4,5,38-41</sup>

Locus	Sequence	Notes
AAVS1-F primer	CTCTAACGCTGCCGTCTCTC	PCR conditions: T <sub>m</sub> = 57 °C, 35 cycles
AAVS1-WT-R primer	GCTTCTCCTCTTGGGAAGTG	WT band: 1273 bp
AAVS1-Targeted-R primer	CGTCACCGCATGTTAGAAGA	Targeted band: 992 bp
T2-Cas9-guide	GGGCCACTAGGGACAGGAT	From Mali <i>et al.</i> , 2013
AAVS1-ZFN-Right	TAGGGACAGGAT	From Hockemeyer <i>et al.</i> , 2009
AAVS1-ZFN-Left	TGGGGTGTCCACC	From Hockemeyer <i>et al.</i> , 2009
AAVS1-TALEN-Right	TCCTAACCACTGTCTTT	From Hockemeyer <i>et al.</i> , 2011
AAVS1-TALEN-Left	CCCCTCCACCCACAGT	From Hockemeyer <i>et al.</i> , 2011

**Table 2. List of primers and SSN targeting sequences.**

Targeting Construct	Number of EGFP+ Colonies	Targeted picked clones (PCR verified)	Previous Reported Correct Targeting Efficiency (by Southern blot)
ZFN	150	86.9% (73.9% het/ 13.0 % homo)	56% (50% het/6% homo)
TALEN	412	91.3% (47.8% het/39.1% homo)	47% (37.5% het/9.3% homo)
CRISPR-Cas9	235	95.7 % (69.5% het/26.3% homo)	unreported

**Table 3. Comparative numbers of EGFP-positive and targeted TALEN, ZFN and CRISPR/Cas9 human stem cell colonies.** PCR verified integrations at the AAVS1 locus for this experiment are compared to Southern blot verified proper single integrations in previous experiments<sup>4,5</sup>.

## Discussion

The method presented here for isolating homogenous populations of gene-edited human pluripotent stem cells is a powerful approach for generating isogenic hPSC lines that differ only at the targeted locus. These cells are an ideal system for probing the mechanisms of human cellular differentiation and development as well as for understanding the pathophysiology of monogenic diseases in a controlled genetic setting. As demonstrated here, it is possible to use three independent SSN design strategies (ZFNs, TALENs, and CRISPR/Cas9) to achieve targeted integration at the AAVS1 locus. Each of these methodologies has its own advantages and disadvantages. A potential advantage of ZFNs and, to some extent, TALENs is their design flexibility, which allows iterative engineering in order to improve the DNA binding domains of individual nucleases<sup>42</sup>. This nuclease optimization could increase the specificity of ZFNs and TALENs beyond what is achievable with the CRISPR/Cas9 system. Such selectivity may be important for clinical applications requiring a high degree of target specificity. The primary advantage of the CRISPR/Cas9 system is its ease of use. Although TALEN and ZFN construction kits have been made available to the public (*i.e.*, through Addgene<sup>21</sup>), CRISPR/Cas9-based SSNs are significantly easier to construct, as the only necessary customization is a 20 base pair



oligonucleotide (when using the px330 plasmid design<sup>14</sup>). This simplicity proves to be advantageous for research labs looking to include genome editing in their studies.

There are alternative transfection techniques, including nucleofection<sup>43</sup>, to create gene-edited hPSC lines; however electroporation has been shown to be consistent and cost effective<sup>4,5,25</sup>. Nucleofection can be used to directly transfect Cas9-guide RNA ribonucleoprotein complexes into the nucleus, increasing the SSN efficiency and fidelity<sup>44</sup>. Growing hPSCs on MEFs is a robust and inexpensive method to maintain hPSCs in a pluripotent state without excessive differentiation. In addition, it allows for the easy isolation of genetically identical colonies. Alternatively, it is possible to culture hPSCs without MEFs, however these culture conditions can be more expensive than feeder based cultures. Furthermore, the whole process is scalable, allowing for the isolation of very rare editing events or the generation of many distinct cell lines in parallel editing experiments.

The protocol described here is robust; however there are several key steps that affect the efficiency with which correctly edited clones can be obtained. The most critical component for this method is having high quality MEFs and drug resistant DR4 MEFs. The survival of single hPSCs is tenuous, and low-quality MEFs will impede the isolation of undifferentiated hPSC lines. Second, the use of Y-27632 is also key to allow single cell survival without generating a selective pressure for cells with an abnormal karyotype<sup>45</sup>. Third, picking well-spaced colonies ensures genetic homogeneity of the derived cell lines. Finally, it is important to prepare glass pipettes so that the opening is small enough to break the colony into many smaller pieces, ensuring that multiple colonies will grow in the new well. This permits the picking of well-spaced subclones for a replica plate to have in culture, leaving the original plate of isolated colonies for genotyping. A challenging part in this protocol is the manual pulling of the glass pipettes; this should be practiced beforehand. It should be noted that there are multiple techniques of clone picking that do not require a glass pipette. Experimenters are encouraged to find the one that works best for them.

There are limitations to this protocol that can be overcome by simple modifications. An experiment intended to only disrupt the locus of interest without repair or one whose repair template does not contain a selection cassette, must use another method of enriching for cells that were edited. Note that the number of colonies that must be picked to find a positive clone increases greatly in the absence of selection. One strategy to improve the efficiency is to co-transfect a non-integrating plasmid that expresses a fluorescent protein. After allowing the cells to recover for two days, targeted cells can be sorted on positive fluorescence using fluorescence-activated cell sorting and replated<sup>29</sup>. This process enriches for cells that have been transfected with the plasmids by electroporation and thereby increases the probability of a concurrent editing event in the cell.

The techniques described here can be extended to use multiple guide RNAs to simultaneously target several loci<sup>14,46,47</sup>. Many protocols have been established to differentiate hPSCs into distinct cell types, allowing various genetic manipulations in cell types of interest<sup>30</sup>. Overall, we have demonstrated the potential for efficient genome editing in hPSCs regardless of SSN choice. We propose that this technique can be adapted to create isogenic hPSC lines that have been gene-edited at any genomic locus.

## Disclosures

The authors declare no competing financial interests.

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